JOURNAL OF MOLECULAR RECOGNITION
J. Mol. Recognit. 2007; **20**: 185–196
Published online 8 May 2007 in Wiley InterScience
(www.interscience.wiley.com) DOI:10.1002/jmr.823

Epitope mapping of the neuronal growth inhibitor Nogo-A for the Nogo receptor and the cognate monoclonal antibody IN-1 by means of the SPOT technique

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Nogo-A is a potent inhibitor of axonal outgrowth in the central nervous system of adult mammals, where it is expressed as a membrane protein on oligodendrocytes and in myelin. Here we describe an attempt to identify linear peptide epitopes in its sequence that are responsible for the interaction either with the Nogo receptor (NgR) or with the neutralizing monoclonal antibody IN-1. Analysis of an array of immobilized overlapping 15 mer peptides covering the entire amino acid sequence of human Nogo-A (1192 residues) revealed a single epitope with prominent binding activity both towards the recombinant NgR and the IN-1 F_{ab} fragment. Further truncation and substitution analysis yielded the minimal epitope sequence 'IKxLRRL' (x≠P), which occurs within the so-called Nogo66 region (residues 1054–1120) of Nogo-A. The bacterially produced Nogo66 fragment exhibited binding activity both for the recombinant NgR and for the IN-1 F_{ab} fragment on the Western blot as well as in ELISA. Unexpectedly, the synthetic epitope peptide and the recombinant Nogo66 showed cross-reactivity with the 8-18C5 F_{ab} fragment, which is directed against myelin oligodendrocyte glycoprotein (MOG) as a structurally unrelated target. On the other hand, the recombinant N-terminal domain of Nogo-A (residues 334–966) was shown to specifically interact on the Western blot and in an ELISA with the IN-1 F_{ab} fragment but not with the recombinant NgR, which is in agreement with previous results. Hence, our data suggest that there is a distinct binding site for the Nogo receptor in the Nogo66 region of Nogo-A, whereas its interaction with NgR is less specific than anticipated before. Although there probably exists a non-linear epitope for the neutralizing antibody IN-1 in the N-terminal region of Nogo-A, which is likely to be accessible from outside the cell, a previously postulated second binding site for NgR in this region (called Nogo-A-24) remains elusive. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: **bacterial expression; Fab fragment; neuronal growth inhibitor; NgR; peptide synthesis; protein–ligand interaction**

Received 2 December 2006; revised 28 February 2007; accepted 1 March 2007

INTRODUCTION

Understanding the mechanisms that underlie the lack of regeneration in the central nervous system (CNS) of higher vertebrates is one of the major challenges in neurobiology. It has been shown that the inability of the axonal tissue to self-regenerate results in part from the existence of inhibitory molecules that are abundant in adult CNS myelin (Woolf and Bloechlinger, 2002; Filbin, 2003; Schwab, 2004).

Essentially three different inhibitory proteins have been identified so far: Nogo-A (Chen *et al.*, 2000; GrandPre *et al.*, 2000; Prinjha *et al.*, 2000), oligodendrocyte-myelin glycoprotein (OMgp), a glycosylphosphatidylinositol-linked protein with a functionally important leucine-rich repeat domain (Wang *et al.*, 2002a; Vourc'h and Andres, 2004), and myelin associated glycoprotein (MAG) (Domeniconi *et al.*, 2002; Liu *et al.*, 2002), a transmembrane protein with several extracellular immunoglobulin type domains. All the three inhibitors seem to exert at least a part of their activity via interaction with the so-called Nogo66/Nogo receptor (NgR) (Fournier *et al.*, 2001; Liu *et al.*, 2002; Wang *et al.*, 2002a; McGee and Strittmatter, 2003; Schwab *et al.*, 2006), a 473 residue glycosylphosphaditylinositol-anchored axon surface protein that presents a large leucine-rich repeat ectodomain (He *et al.*, 2003). Hence, NgR and its ligands, in particular Nogo-A, constitute potential targets for therapeutic intervention of spinal cord injuries (Lee *et al.*, 2003; McKerracher and David, 2004; Schwab, 2004).

In vitro, the strong inhibitory activity of CNS myelin extracts can be partially neutralized by antibodies directed against Nogo-A (Brösamle *et al.*, 2000; Buffo *et al.*, 2000;

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Abbreviations used: Amp, ampicillin; aTc, anhydrotetracycline; CNS, central nervous system; GST, glutathione S-transferase; IMAC, immobilized metal affinity chromatography; MAG, myelin associated glycoprotein; MOG, myelin oligodendrocyte glycoprotein; NgR, Nogo/Nogo66 receptor; OMgp, oligodendrocyte-myelin glycoprotein.

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Wiessner *et al.*, 2003), by soluble NgR fragments (Fournier *et al.*, 2002), and by NgR-blocking peptides (GrandPre *et al.*, 2002). The Nogo-A specific monoclonal antibody IN-1 (Caroni and Schwab, 1988) was shown in several rat models of nerve injury to improve the axonal outgrowth and functional recovery *in vivo* when infused at the lesion site (Schnell and Schwab, 1990; Bregman *et al.*, 1995; Thallmair *et al.*, 1998).

Nogo-A, originally dubbed NI-220/250 (Huber and Schwab, 2000), was discovered in bovine spinal cord as a predominant protein expressed on the surface of oligodendrocytes and myelin, which suppresses the axonal growth in neuronal cell culture assays. The corresponding cDNAs were subsequently identified for rat and man (Chen *et al.*, 2000; GrandPre *et al.*, 2000; Prinjha *et al.*, 2000). Nogo-A is the largest known splice variant of the *nogo* gene, which can give rise to three major isoforms (Chen *et al.*, 2000), all constituting members of the reticulon family of proteins (Fournier *et al.*, 2001): Nogo-A is mainly expressed in the adult mammalian CNS, both on oligodendrocytes and certain neuronal populations; the Nogo-B splice form is found in many tissues and cell types including adult neurons; Nogo-C is predominantly expressed in muscles (Huber *et al.*, 2002; Hunt *et al.*, 2002; Tozaki *et al.*, 2002; Wang *et al.*, 2002b). The physiological functions of Nogo-B and Nogo-C are still a matter of debate.

Human Nogo-A is encoded by altogether nine exons (Oertle *et al.*, 2003a), the first three of which are specific for the A isoform and give rise to a presumed N-terminal extracellular region of ca. 1000 amino acids (Oertle *et al.*, 2003b; Fiedler *et al.*, 2002) which exert neuronal growth inhibitory activity (Chen *et al.*, 2000; Oertle *et al.*, 2003b). The C-terminal moiety comprises two hydrophobic transmembrane or at least membrane-associated regions that encompass a short extramembrane domain called Nogo66, which is common to Nogo-A, -B, and -C (Prinjha *et al.*, 2000). Nogo66 is detectable on the surface of oligodendrocytes and was demonstrated to bind NgR (GrandPre *et al.*, 2000; Fournier *et al.*, 2001; Oertle *et al.*, 2003b). Furthermore, peptides derived from Nogo66 were shown to block the inhibitory effect of myelin on the neurite growth (GrandPre *et al.*, 2002).

The characteristic N-terminal domain of Nogo-A was demonstrated to inhibit the axonal outgrowth and spreading of non-neuronal cells (Chen *et al.*, 2000; Fournier *et al.*, 2001). A central segment called Δ20 (residues 544 to 725 in rat; 567 to 748 in man) appeared to be the predominant inhibitory substrate for primary neurons, PC12 cells as well as fibroblasts (Oertle *et al.*, 2003b). Although the inhibition of cell spreading mediated by N-terminal Nogo-A fragments was shown to be NgR-independent (Niederost *et al.*, 2002; Oertle *et al.*, 2003b), a region at its very C-terminus, dubbed Nogo-A-24 (residues 995 to 1018 in the human protein), was recently reported to interact with NgR (Hu *et al.*, 2005; Schwab *et al.*, 2006). These findings suggest that a systematic analysis of the Nogo-A sequence for NgR binding sites should be useful for better understanding the mechanisms of their mutual interaction on a molecular level.

The mouse monoclonal IgM/κ antibody IN-1 has so far been the most thoroughly investigated example among several Nogo-A-specific antibodies which neutralize the growth inhibitory activity of CNS myelin (Caroni and Schwab, 1988; Brösamle *et al.*, 2000; Chen *et al.*, 2000; Fiedler *et al.*, 2002). IN-1 was originally raised against the Nogo-A protein extracted from SDS-polyacrylamide gels of rat spinal cord myelin (Caroni and Schwab, 1988). Later, its variable domain cDNAs were cloned and the corresponding recombinant F_{ab}-fragment was produced in *E. coli* (Bandtlow *et al.*, 1996). Similarly to the intact myeloma protein this partially humanized F_{ab}-fragment was shown to promote the regeneration of corticospinal axons in adult rats after spinal cord lesion *in vivo* (Brösamle *et al.*, 2000).

Unfortunately, IN-1 as well as its recombinant F_{ab}-fragment exhibit rather low antigen affinity, suggesting that this antibody has originated from an early immune response (Bandtlow *et al.*, 1996). For this reason, an engineered IN-1 F_{ab}-fragment with improved affinity towards the bacterially produced N-terminal rat Nogo-A domain was generated and appeared to possess enhanced neutralizing activity (Fiedler *et al.*, 2002). An important next step to assist the development of even better antagonistic antibodies would be the identification of the cognate epitope on the rather large Nogo-A antigen, which also seems to be involved in its neuronal growth inhibitory activity.

Taken together, plenty of data indicate that understanding the structure and function both of Nogo-A and of NgR as well as their mutual molecular interaction should be pivotal for the development of improved strategies for the medical treatment of CNS injuries, and the antibody IN-1 may be a promising candidate in this respect. Consequently, in this study we present an attempt to characterize the interactions with NgR as well as with the IN-1 F_{ab} fragment via systematic mapping of potential linear epitopes in the Nogo-A sequence.

MATERIALS AND METHODS

Vector construction for the bacterial production of NgR fragments

The extracellular region of mature NgR (residues 26–447) was amplified via PCR from human brain cDNA (kindly provided by E. Meindl, MPI für Neurobiologie, Martinsried, Germany) with *Taq* DNA polymerase High Fidelity (Roche Diagnostics, Mannheim, Germany) using the primers 5′-TGG GGA TCC CAT GCC CAG GTG CCT GCG TA-3′ (*Bam*HI restriction site underlined) and 5′-GGG AAT TCA GCC TTC TGA GTC ACC AGT CC-3′ (*Eco*RI restriction site underlined). The single PCR product was digested with *Bam*HI and *Eco*RI (both from New England Biolabs, Bad Schwalbach, Germany), purified via agarose gel electrophoresis, and inserted into the multiple cloning site of pGEX5X (Amersham Biosciences, Freiburg, Germany). The resulting vector, pGEX-NgR, encodes a fusion protein of NgR with (dimeric) gluthathione-S-transferase (GST) from *Schistosoma japonicum* at its N-terminus and a total mass of 72 kDa per polypeptide chain. Subsequently, the expression vector pASK111-TrxNgR was constructed by amplifying the NgR gene from pGEX-NgR with *Pfu* DNA polymerase (Stratagene, Amsterdam, Netherlands) using the phosphorothioate primers 5′-CTG CAG GCC GAT GGG GCC CTT GGC CCA TGC CCA GGT GCC TG p(S)T-3′ (*Sfi*I restriction site

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DOI: 10.1002/jmr

underlined) and 5′-GCT GCC TTC TGA GTC ACC AGT C p(S)T-3′. The unique PCR product was digested with *Sfi*I, purified, and inserted into pASK111-TrxDkk—a derivative of pASK111 (Vogt and Skerra, 2001) encoding *E. coli* thioredoxin–which was cut with *Bgl*I (at the 3′ end of the *trx*A gene, generating a compatible restriction site with *Sfi*I) as well as *Eco*47III (generating a blunt end directly upstream of the *Strep*-tag), yielding pASK111-TrxNgR. This plasmid encodes a fusion protein of NgR with thioredoxin (LaVallie *et al.*, 1993) at its N-terminus and the *Strep*-tag (Skerra and Schmidt, 2000) at its C-terminus, having a total mass of ca. 60 kDa. A vector encoding just the ligand-binding domain, pASK111-TrxNgRΔ, was constructed from pASK111-TrxNgR by deleting the C-terminal 136 codons from the cloned NgR gene via site-directed mutagenesis (Geisselsoder *et al.*, 1987) using the oligodeoxynucleotide 5′-CGG GTG ACG CCA AGC GCT AGC GCA GCC CTG CAG GTC-3′. Cys residues at positions 80 and 140 (with respect to the reading frame on the original NgR cDNA) were replaced by Ser and Asn, respectively, via site-directed mutagenesis using the oligodeoxynucleotides 5′-GGT GAG GTT GCG GGA GGC ACG GAA GC-3′ and 5′-CTC CTG CAG GCC GTT GCG GTC CAG GTG-3′. All plasmid constructions and mutagenesis experiments were confirmed by restriction analysis, followed by DNA sequencing on an ABI Prism Genetic Analyser 310 with the BigDye Terminator Kit (Applied Biosystems, Weiterstadt, Germany).

Bacterial expression of NgR fragments

E. coli JM83 (Yanisch-Perron *et al.*, 1985) transformed with pASK111-TrxNgR or pASK111-TrxNgRΔ was grown in shake flasks containing 2 l LB medium (Sambrook *et al.*, 1989) supplemented with 100 mg/l ampicillin (Amp) at 37°C. Foreign gene expression was induced at an optical density at 550 (OD_{550}) of 0.5 by addition of 200 μg/l anhydrotetracycline (aTc; Acros Organics, Geel, Belgium) as described (Skerra, 1994b). After 3 h induction the bacteria were harvested by centrifugation. For preparation of a cytoplasmic extract the bacteria were resuspended in 20 ml of 150 mM NaCl, 1 mM EDTA, 0.1 M Tris/HCl pH 8.0 and homogenized three times in a French Pressure Cell (G. Heinemann Ultraschall- und Labortechnik, Schwäbisch Gmünd, Germany) at 15 000 psi. After centrifugation (20 000 g, 30 min) the sedimented inclusion bodies were resuspended in PBS (4 mM KH_2PO_4, 16 mM Na_2HPO_4, 115 mM NaCl) containing 6 M Gdn/HCl to yield a protein concentration of 10 mg/ml. The inclusion bodies containing TrxA-NgRΔ were refolded by 100 fold dilution into 55 mM Tris/HCl pH 8.2, 264 mM NaCl, 11 mM KCl, 2.2 mM $MgCl_2$, 2.2 mM $CaCl_2$, 0.3 mM lauryl maltoside, 440 mM sucrose, 550 mM L-arginine, 1 mM DTT at 4°C. Aggregates were removed by centrifugation and the resulting protein solution was dialysed against 150 mM NaCl, 1 mM EDTA, 0.1 M Tris/HCl pH 8.0. TrxA-NgRΔ was purified by means of the *Strep*-tag using streptavidin affinity chromatography (Skerra and Schmidt, 2000). In contrast to TrxA-NgRΔ, the full-length fusion protein remained insoluble after refolding. For preparation of the GST-NgR fusion protein *E. coli* strains JM83, BL21 or Origami (Novagen, Madison, WI, USA) were transformed with pGEX-NgR and grown in shake flasks containing 2 l LB medium supplemented with 100 mg/l Amp at 30°C or 37°C. Foreign gene expression was induced at $OD_{550} = 0.5$ by addition of 0.1 mM IPTG (Sigma, Deisenhofen, Germany). After 3 h of induction the bacteria were harvested by centrifugation and a cytoplasmic extract was prepared as described above. While most of the recombinant protein was found aggregated in inclusion bodies, just a minor fraction of GST-NgR could be detected in the soluble fraction, which appeared to be tightly associated with the molecular chaperone GroEL. Several attemps to purify GST-NgR from the soluble protein fraction or to refold it from the deposited inclusion bodies remained unsuccessful so that this fusion protein was not used for further experiments.

Cloning and expression of the Nogo66 fragment

The Nogo66 fragment of rat, which differs just in a single residue from the corresponding human fragment (Ser1073 in the rat sequence instead of chemically reactive Cys1101 for the human protein), was amplified from the cloned cDNA (Chen *et al.*, 2000) via PCR with *Pfu* DNA polymerase using the phophorothioate primers 5′-TGA CTA TCC ATA TGA GGA TAT ATA AGG GCG TGA TCp(S)T-3′ (*Nde*I restriction site underlined) and 5′-GCT CAG GGA ATC AAC TAA ATC ATCp(S)T-3′. The PCR product was digested with *Nde*I, purified, and inserted into a derivative of pRSET5a (Schoepfer, 1993) encoding the extracellular domain of CD16 with a C-terminal His_6 tag (A. Skerra and coworkers, unpublished), which had been cut with *Nde*I (at the translational start site) and *Eco*47III (directly upstream of the His_6 tag). The resulting plasmid, pN66, leads to the cytoplasmic production of rat Nogo66 fused with a His_6 tag at its C-terminus, having a total mass of ca. 8.5 kDa. *E. coli* BL21 transformed with pN66 was grown and harvested as described in the preceding section, whereby gene expression was induced with 0.5 μM isopropyl-β-D-thiogalactopyranoside (IPTG) over night. Inclusion bodies were again solubilized at a concentration of 10 mg/ml in PBS containing 6 M Gdn/HCl. The recombinant protein was purified via the His_6 tag (Skerra, 1994a) by means of immobilized metal affinity chromatography (IMAC) on Zn(II)-charged IDA-Sepharose (Chelating Sepharose Fast Flow; Amersham Biosciences) under denaturing conditions using 6 M Gdn/HCl, 0.1 M Tris/HCl pH 7.5 as chromatography buffer and 300 mM imidazole/HCl in the same buffer for batch elution. The purified protein (1 mg/ml) was refolded by 100 fold dilution into PBS at 4°C. Finally, aggregates were removed by centrifugation and, if necessary, the protein solution was concentrated by ultrafiltration.

Production of the recombinant N-terminal Nogo-A fragment, NogoAn

The cDNA for the soluble N-terminal extramembrane region of Nogo-A (residues 334–966 of the 1192 amino acid human full length protein), dubbed NogoAn (Zander *et al.*, 2007), which had been cloned on the vector pASK75-strepII

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DOI: 10.1002/jmr

(Skerra, 1994b), was produced in the *E. coli* strain HM125 (Meerman and Georgiou, 1994). Transformed cells were grown at 22°C in 2 l LB medium containing 100 mg/l Amp. Gene expression was induced by the addition of 200 µg/l aTc at $OD_{550} = 0.5$ for 2.5 h. The cells were harvested by centrifugation and proteins were extracted from the periplasm by incubation for 30 min on ice with 20 ml of 0.5 M sucrose, 1 mM EDTA, 0.1 M Tris HCl pH 8.0 containing 100 µg/ml lysozyme. The resulting spheroplasts were sedimented by centrifugation and the supernatant was recovered. NogoAn was purified from this periplasmic protein extract via the *Strep*-tag II fused to its C-terminus using streptavidin affinity chromatography. Elution was effected under native conditions in the presence of D-desthiobiotin (Skerra and Schmidt, 2000).

Production of recombinant F_{ab} fragments

The recombinant F_{ab} fragment of the antibody IN-1 (Bandtlow *et al.*, 1996), its engineered version IN-1.II.I.8 (Fiedler *et al.*, 2002), and the MOG-specific 8-18C5 F_{ab} fragment (Breithaupt *et al.*, 2003) were used in this study. The variant II.I.8 was derived from the original IN-1 F_{ab} fragment via *in vitro* affinity maturation and carries five side chain substitutions within CDR-L3 (Fiedler *et al.*, 2002). All F_{ab} fragments were produced in the periplasm of *E.coli* JM83 as previously described using the vector pASK107 (Fiedler *et al.*, 2002). The F_{ab} fragments were purified via the *Strep*-tag II attached to the C-termini of their heavy chains using streptavidin affinity chromatography (Skerra and Schmidt, 2000).

SPOT synthesis of immobilized peptide arrays and identification of Nogo-A epitope peptides

Arrays of 394 overlapping 15 mer peptides covering the entire amino acid sequence of human Nogo-A as well as specific arrays for truncation and substitution analysis were automatically prepared according to standard SPOT synthesis protocols (Frank, 2002) using a SPOT synthesizer (Abimed, Langenfeld, Germany). Briefly, the peptides were synthesized on an amino-functionalized cellulose membrane as distinct spots. A β-alanine dipeptide spacer was inserted between the C-terminus of each peptide and the membrane support. The peptide was extended stepwise by using standard fluorenylmethoxycarbonyl solid-phase peptide synthesis, followed by cleavage of the side chain protecting groups under trifluoroacetic acid conditions. Sequence files were generated with the software DIGEN (Jerini, Berlin, Germany). All peptides were N-terminally acetylated.

To detect binding activity on the SPOT membranes two different protocols were followed. For signal development with horse radish peroxidase (HRP) the membrane was first blocked over night at 4°C with 10% w/v 'blocking reagent' (Roche Diagnostics, Penzberg, Germany) in membrane blocking solution (MBS; 50 mM Tris/HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 0.05% v/v Tween-20, 1% w/v sucrose). After washing for 10 min in TBS/T (50 mM Tris/HCl pH 8.0, 137 mM NaCl, 2.7 mM KCl, 0.05% v/v Tween-20) the membrane was incubated for 1 h at room temperature with a 2 µM solution of the IN-1 F_{ab} fragment in TBS/T. Then, the membrane was washed three times with TBS/T and incubated for 1 h with an anti-*Strep*-tag II antibody HRP conjugate (IBA, Göttingen, Germany) at a dilution of 1/1000 in MBS containing 10% w/v 'blocking reagent'. Signals were developed using the SuperSignal chemiluminescence detection system (Pierce, Rockford, Il) by exposing the membrane to Hyperfilm (Amersham Biosciences). Alternatively, signal development with alkaline phosphatase was performed according to a published procedure (Frank and Overwin, 1996). Briefly, the membane was blocked in MBS and incubated with 2 µM protein solutions of the F_{ab} fragments or of TrxA-NgRΔ in MBS, followed by incubation with StrepTactin-alkaline phosphatase (AP) conjugate (IBA) in MBS at a dilution of 1:1000. Signals were developed in 10 ml of 137 mM NaCl, 2.7 mM KCl, 0.05 mM $MgCl_2$, 10 mM Na-citrate pH 7.0 with the addition of 40 µL BCIP (60 mg/ml 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt in DMF) and 60 µL MTT (50 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide in 70% v/v DMF). The membrane was regenerated with 2-mercaptoethanol/SDS reagent according to the published protocol and the quantitative removal of bound proteins was confirmed by subsequent incubation with the StrepTactin conjugate alone, followed by signal development.

ELISA

To determine binding activities of recombinant F_{ab} fragments and NgR towards Nogo66 or NogoAn a microtitre plate was coated with 50 µL per well of either 1 µg/ml Nogo66 or 100 µg/ml NogoAn in PBS for 2 h and blocked with 3% w/v bovine serum albumin (BSA; Fraction V, 98% purity, Sigma Aldrich, Munich, Germany) in PBS/T (PBS containing 0.5% v/v Tween) for 1 h. The bacterially produced F_{ab} fragments IN-1, 8-18C5, and IN-1.II.1.8 as well as the recombinant TrxA-NgRΔ fusion protein were labeled with digoxigenin-3-*O*-methylcarbonyl-ε-aminocaproic acid *N*-hydroxy-succinimide (Roche Diagnostics, Penzberg, Germany) at a molar ratio of 2:1 as previously described (Schlehuber *et al.*, 2000). The digoxigenin-labelled F_{ab} fragments or NgR were applied as dilution series in PBS/T and incubated for 1 h. Bound protein was detected with anti-digoxigenin F_{ab} fragment conjugated with alkaline phosphatase (Roche Diagnostics) at a dilution of 1:2500. Signals were developed with *p*-nitrophenyl phosphate, quantified at 405 nm in a SpectraMax 250 reader (Molecular Devices, Sunnyvale, CA), and evaluated as described (Voss and Skerra, 1997).

Western blotting

For Western blotting of NogoAn and Nogo66, proteins were separated by 0.1% w/v SDS 10% w/v PAGE (Fling and Gregerson, 1986) and electro-transferred onto a PVDF membrane (Millipore, Schwalbach, Germany), which was subsequently blocked with 3% w/v low-fat milk powder (Vitalia, Sauerlach, Germany) in PBS/T for 1 h. After washing with PBS/T the membrane was incubated with 100 nM of digoxigenin-labelled F_{ab} fragments or the NgR

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DOI: 10.1002/jmr

fusion protein in PBS/T, followed by the anti-digoxigenin F_{ab} fragment-alkaline phosphatase conjugate at a dilution of 1:1000. After washing with PBS/T and PBS, signals were developed (Schlehuber *et al.*, 2000) in 10 ml AP-buffer (100 mM Tris/HCl pH 8.8, 100 mM NaCl, 5 mM $MgCl_2$) by adding 60 µl BCIP (50 mg/ml in DMF) and 10 µL NBT (75 mg/ml nitro-blue tetrazolium chloride in 70% v/v DMF). Alternatively, the blotted proteins were directly stained by incubation for 5 min in a 1 mg/ml solution of Ponceau S (Sigma), followed by destaining as appropriate in water.

Fluorescence titration

Fluorescence titration of recombinant F_{ab} fragments or the NgR fusion protein with a synthetic epitope peptide was carried out as described (Voss and Skerra, 1997) in an LS 50 B fluorimeter (Perkin-Elmer, Norwalk, CT) using a 1 cm^2 quartz cuvette thermostated at 25°C. Wavelengths for excitation and emission were set to 280 and 340 nm, respectively. Aliquots of a 0.5 mM stock solution of the synthetic Nogo66 peptide oAbz-TIKELRRLFL-NH_2 (Peptide Speciality Laboratories, Heidelberg, Germany) were successively added to 2 ml of a 1 µM solution of the purified recombinant protein in PBS, and the fluorescence intensity was measured at each step. A slight volume increase during the titration (2% in total) was neglected. The data were normalized to an initial fluorescence of 100% and fitted by non-linear least squares regression according to the Law of Mass Action for bimolecular complex formation as previously described (Vogt and Skerra, 2001).

RESULTS

Bacterial production of Nogo-A, NgR, and F_{ab} fragments

For this study on the interaction between Nogo-A and its presumed cellular receptor as well as cognate antibody fragments, several recombinant protein reagents were produced in *E. coli* (Figure 1). The human N-terminal Nogo-A fragment (residues 334–966, SWISS-PROT entry Q9NQC3), dubbed NogoAn (Zander *et al.*, 2007), was prepared as a soluble protein via secretion into the periplasm of *E. coli*—similarly as previously described for a fragment of rat Nogo-A (Fiedler *et al.*, 2002)—and purified to homogeneity by means of the *Strep*-tag II (Skerra and Schmidt, 2000) fused to its C-terminus. The human Nogo66 domain (residues 1054–1120), which was fused at its C-terminus with the His_6-tag, was overexpressed in the cytoplasm of *E. coli*, solubilized from inclusion bodies with Gdn-HCl, purified via IMAC, and refolded in PBS.

The recombinant F_{ab} fragments of the Nogo-A specific monoclonal antibody IN-1 (Bandtlow *et al.*, 1996) as well as its engineered version II.I.8 (Fiedler *et al.*, 2002) were produced by secretion into the periplasm of *E. coli* (Skerra, 1994a) and purified to homogeneity via the *Strep*-tag II, which was in each case attached to the C-terminus of the heavy chain. In addition, the F_{ab} fragment of the monoclonal antibody 8-18C5 specific for the myelin oligodendrocyte glycoprotein (MOG), a type I transmembrane protein with an immunoglobulin-like extracellular region (Breithaupt *et al.*, 2003), was produced in *E. coli* using the same strategy to serve as a negative control.

The full-length ectodomain of the mature Nogo receptor, NgR (residues 26–447, SWISS-PROT entry Q9BZR6; without the signal peptide and the GPI-linker), was cloned from human brain cDNA. Initially, an attempt was made to produce it in *E. coli* as a fusion protein with GST according to a published strategy (Fournier *et al.*, 2001). Unfortunately, we could not isolate a truly soluble fusion protein from the bacterial cell extract and, furthermore, refolding from the resulting inclusion body protein failed. Similar negative experiences in the production of soluble Nogo receptor protein, either in the bacterial cytoplasm, periplasm or by refolding from aggregates, were recently reported by others (Schimmele *et al.*, 2005).

In a second attempt, we truncated the recombinant Nogo receptor to its extracellular binding domain, comprising residues 26–310, as apparent from its crystallographic analysis (He *et al.*, 2003; Barton *et al.*, 2003). In addition, the two unpaired Cys residues no. 80 and 140 were substituted by Ser and Asn, respectively. Finally, to promote unimolecular folding of the recombinant protein, NgR was expressed as a fusion protein with *E. coli* thioredoxin at its

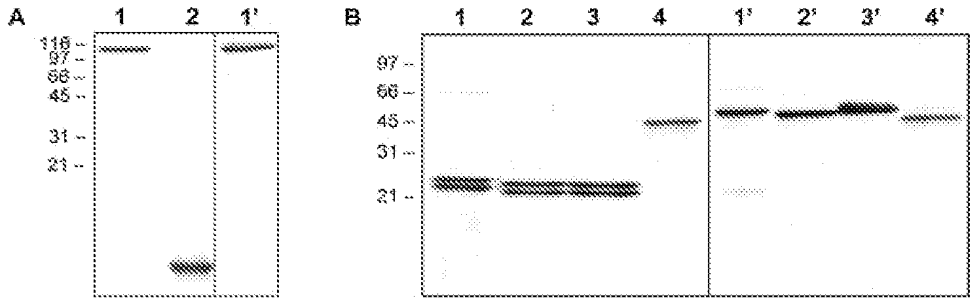

Figure 1. Characterization of the recombinant proteins prepared in this study. (A) Coomassie-stained SDS-PAGE (15% w/v) of the bacterially produced NogoAn and Nogo66 fragments. Lanes: 1, NogoAn; 2, Nogo66; 1′, NogoAn under non-reducing conditions. (B) Coomassie-stained SDS-PAGE (15% w/v) of the bacterially produced F_{ab} fragments as well as the recombinant Nogo receptor fusion protein. Lanes: 1, 8-18C5 F_{ab} fragment; 2, IN-1 F_{ab} fragment; 3, IN-1 variant II.I.8 F_{ab} fragment; 4, TrxA-NgRΔ; 1′ - 4′, the same proteins under non-reducing conditions. Marker sizes are indicated in kDa.

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DOI: 10.1002/jmr

N-terminus, which is known to promote solubility (LaVallie *et al.*, 1993) and also constitutes a monomeric fusion partner, in contrast with the homodimeric GST (Tudyka and Skerra, 1997). Again, expression of the truncated TrxA-NgR fusion protein (TrxA–NgRΔ) in the *E. coli* cytoplasm did not yield soluble protein but gave rise to inclusion bodies. However, these could be refolded with good yields (for details see Materials & Methods) and the soluble NgR fusion protein with thioredoxin was successfully purified to homogeneity via the *Strep*-tag fused to its C-terminus (Figure 1).

Identification of Nogo-A epitopes for the antibody IN-1 as well as potential binding sites for the Nogo receptor

To determine potential linear binding sites in the amino acid sequence of human Nogo-A an array of overlapping 15 mer peptides—each shifted by 3 amino acids—was synthesized on a cellulose membrane, whereby the C-termini were covalently fixed to the support, using the SPOT technique (Frank, 2002). Both the recombinant IN-1 F_{ab} fragment (Bandtlow *et al.*, 1996) and its engineered variant II.1.8 (Fiedler *et al.*, 2002), which were previously shown to recognize the N-terminal domain of Nogo-A, were first used to probe this peptide array (Figure 2). The recombinant F_{ab} fragment 8-18C5 (Breithaupt *et al.*, 2003), which has the same format as the IN-1 F_{ab} fragment but differs in its variable regions, served as negative control.

Both the conventional and the engineered IN-1 F_{ab} fragment showed strong binding signals to peptides nos. 366–368 (Figure 2A and B; data for the IN-1 II.1.8 F_{ab} fragment not shown), covering the amino acid stretch LGHVNSTIKELRRLFLVDDLV (residues 1096–1116), which is part of the so-called Nogo66 domain (comprising residues 1055–1120) (GrandPre *et al.*, 2000). When the same membrane was probed with the MOG specific F_{ab} fragment 8-18C5 (Figure 2C) a very similar pattern for peptides no. 366 and 367 was observed, although with slightly weaker intensities. A control experiment that was carried out just with the StrepTactin–AP conjugate, which was employed for detection of the antibody fragments via the *Strep*-tag II, did not reveal significant signals in this region (not shown), whereas some scattered signals (e.g., for peptides no. 9, 238, and 291) that appeared on the peptide membrane were obviously due to cross-reaction with this reagent. Consequently, there was no specific signal for a linear peptide epitope detectable within the N-terminal extramembrane domain of Nogo-A.

Based on the strong binding signals of several F_{ab} fragments for a sequence stretch within the Nogo66 region we searched for binding sites for the Nogo receptor, NgR, which was discovered via its interaction with Nogo66 (Fournier *et al.*, 2001), in the entire Nogo-A sequence. When using its recombinant fusion protein, TrxA-NgRΔ, as a probe for the Nogo-A peptide SPOT membrane—applying similar conditions as for the recombinant F_{ab} fragments before—strong binding signals were again detected for peptides no. 366–368 and also, with decreasing intensity, for peptides no. 369 and 370 (Figure 2D). This result suggests a binding site in common with the three F_{ab} fragments as described above. Notably, the NgR fusion protein also gave rise to elevated background signals for peptides corresponding to the two hydrophobic transmembrane regions of Nogo-A (peptides nos. 337–341, residues 1009–1034, and peptides nos. 375–379, residues 1123–1149). However, no significant binding signals were detected within the N-terminal extramembrane region of Nogo-A.

Substitutional and length analysis of the Nogo-A peptide epitope

Based on the pronounced binding signals of a stretch of consecutive amino acids within the Nogo66 domain both for the F_{ab} fragments as well as for the recombinant NgR, a substitutional analysis of the predominant peptide no. 367 was performed. In the corresponding sequence 'VNSTI-KELRRLFLVD' each position was systematically replaced by all 20 L-amino acids and, subsequently, its N- and C-termini were truncated in a step-wise fashion (Figure 3).

As result, the core sequence 'IKELRRL', which is common to peptides no. 366, 367, 368, appeared for TrxA-NgRΔ (Figure 3) and, consistently, also for the F_{ab} fragments IN-1 and 8-18C5 (data not shown). In addition, the substitutional analysis revealed that the sequence of the core epitope was sensitive for side chain replacements (Figure 3A). The only exception was Glu at position 3, which could be substituted by all other amino acids apart from Pro without significant loss of binding activity.

Recognition of recombinant Nogo-A fragments by the IN-1 F_{ab} fragment on a Western blot

The binding activity of the recombinant F_{ab} fragments and the NgR fusion protein towards the bacterially produced N-terminal domain of Nogo-A, NogoAn, as well as the recombinant Nogo66 was further investigated on a Western blot. Purified NogoAn and Nogo66 were applied to SDS-PAGE and transferred to a PVDF membrane. The membrane was subsequently incubated with the digoxigenin-labeled IN-1 F_{ab} fragment, the 8-18C5 F_{ab} fragment or TrxA-NgRΔ and bound proteins were detected by means of an anti-digoxigenin F_{ab} fragment-alkaline phosphatase conjugate (Figure 4).

As result, the blotted N-terminal Nogo-A fragment was specifically recognized by the IN-1 F_{ab} fragment, which is in agreement with previous findings (Fiedler *et al.*, 2002). No signal appeared for the MOG specific 8-18C5 F_{ab} fragment, which served as negative control. Binding activity was also not detectable for the bacterially produced Nogo receptor, which had to be anticipated because NgR was previously shown to be directed against the Nogo66 region (Fournier *et al.*, 2001).

Contrasting with these findings for NogoAn, the blotted Nogo66 domain yielded staining signals not only for the NgR fusion protein but also for the other reagents tested in this study, that is, the IN-1 F_{ab} fragment and the 8-18C5 F_{ab} fragment. The engineered version II.1.8 of the IN-1 F_{ab} fragment gave rise to a similar pattern (data not shown). This result confirmed our observations from the epitope mapping experiment, where especially peptides within the Nogo66

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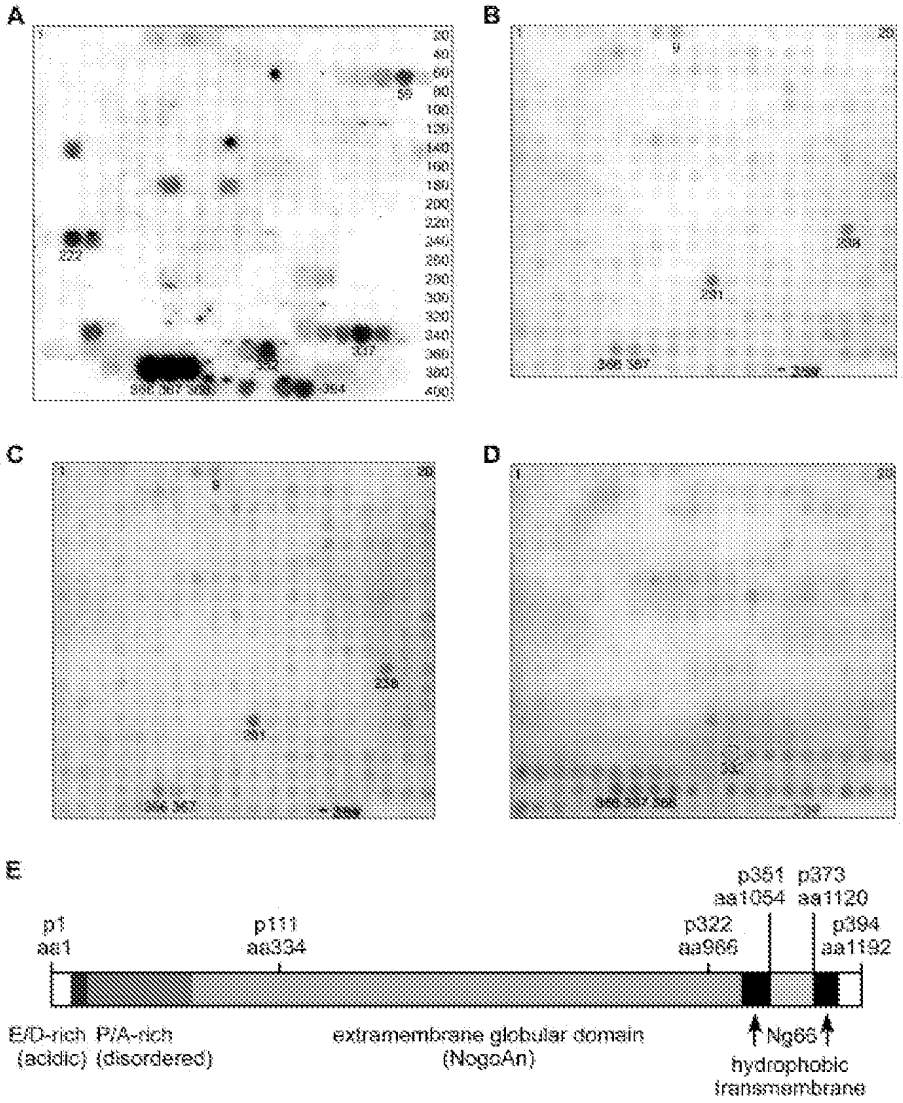

Figure 2. Epitope analysis of human Nogo-A using a complete set of immobilized 15 mer peptides prepared by SPOT synthesis, each one shifted by three amino acids, covering the entire primary sequence. Thus, the peptide on the first spot at the upper left (labeled 1) comprises residues 1–15 of Nogo-A, the second one 4–18 etc., resulting in altogether 394 spots for the 1192 residue protein. (A) The membrane was probed with the recombinant IN-1 F_{ab} fragment (2 μM) and stained with a secondary (anti-*Strep*-tag II) antibody HRP conjugate. Signals were developed using the SuperSignal chemiluminescence detection system. The three spots giving rise to the most intense signals correspond to peptides no. 366 (LGHVNSTIKELRRLF), 367 (VNSTIKELRRLFLVD), and 368 (TIKELRRLFLVDDLV), which are located in the Nogo66 region. Much less intense signals could be detected at spots no. 59 (APKRRGSSGSVDETL), 222 (SVSLKKVSGIKEEIK), 337 (LYWRDIKKTGVVFGA), 352 (FRIYKGVIQAIQKSD), and 394 (AKIQAKIPGLKRKAE). However, these signals seemed not to be specific for the IN-1 F_{ab} fragment because they were no longer detectable when another secondary reagent was used (see next panel). (B) A fresh membrane with the same array was again probed with the recombinant IN-1 F_{ab} fragment (2 μM) but stained with StrepTactin–AP conjugate (also recognizing the *Strep*-tag II fused to the heavy chain of the antibody fragment) using MTT and BCIP as chromogenic substrates. Again, peptides no. 366 and 367 gave rise to signals. In contrast, the spots no. 9, 238, and 291 also appeared when the same membrane was probed with the StrepTactin–AP conjugate alone (not shown) and thus seemed to be unspecific. (C) The membrane was probed with the 8-18C5 F_{ab} fragment (2 μM), which has the same format as the IN-1 F_{ab} fragment but differs in its variable regions and binds to an unrelated antigen (Breithaupt *et al.*, 2003). The membrane was developed as in (B). (D) Epitope mapping with the recombinant NgR fusion protein (TrxA-NgRΔ; 2 μM), followed by detection with StrepTactin–AP and MTT/BCIP as in (B) and (C). Again, peptides nos. 366–368 gave rise to prominent signals even though accompanied by a generally higher background, especially for those peptides corresponding to the two transmembrane segments flanking both sides of the Nogo66 region. Peptide no. 332 (AIFSAELSKTSVVDL; residues 994–1008), which reveals a comparably negligible signal, was recently postulated to be a second binding site for NgR outside the Nogo66 region (Hu *et al.*, 2005) and is labeled. (E) Schematic representation of Nogo-A with its different domains (aa denotes residue numbers while p denotes peptide numbers corresponding to those on the SPOT array).

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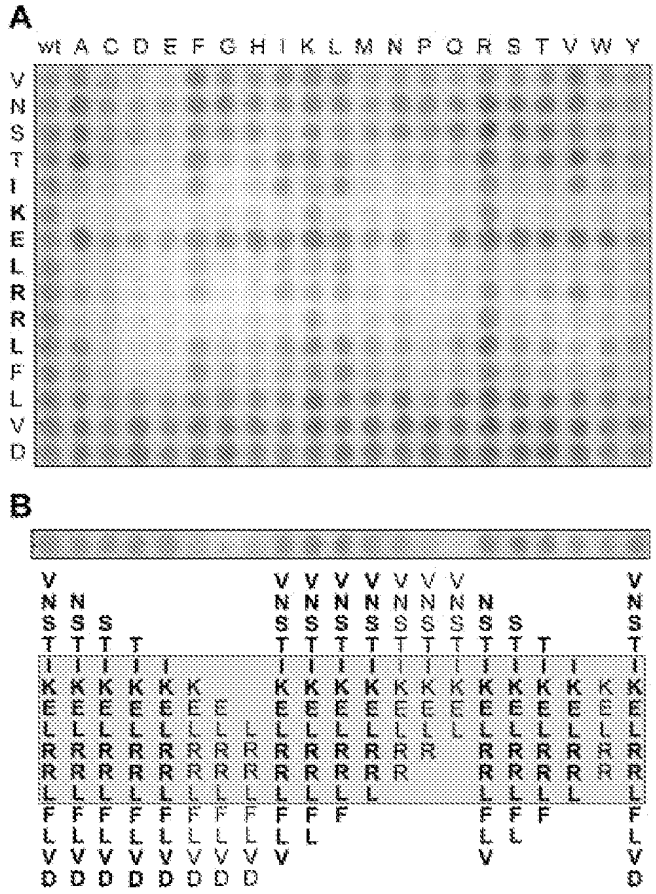

Figure 3. Substitution and length analysis of the Nogo-A epitope for the recombinant NgR fusion protein as identified in Figure 2. (A) Each residue of peptide no. 367, whose sequence is given from top to bottom on the left margin, was systematically substituted by all 20 L-amino acids (as indicated at the top of each column). The membrane was probed with TrxA-NgRΔ (2 μM) and developed as in Figure 2D. (B) Peptide no. 367 was shortened in a step-wise fashion—on the same sheet of membrane as used for (A)—from both termini, whereby the corresponding peptide sequences are indicated below the spots. Amino acids comprising the minimal epitope are highlighted.

region exhibited strong binding activity towards all F_{ab} fragments and the recombinant NgR.

Epitope binding studies by ELISA and fluorescence titration

To investigate interactions between the recombinant F_{ab} fragments or NgR and the two bacterially produced antigen fragments under native conditions, a microtitre plate was coated with NogoAn and Nogo66, respectively, blocked, and incubated with the purified, digoxigenin-labelled F_{ab} fragments or TrxA-NgRΔ. Bound protein was again detected by means of an anti-digoxigenin F_{ab} fragment-alkaline phosphatase conjugate (Figure 5).

Indeed, the engineered IN-1 F_{ab} fragment H.I.8 showed significant binding activity towards NogoAn, which is in agreement with previous results (Fiedler *et al.*, 2002) and with the Western blotting experiments described above. In contrast, the 8-18C5 F_{ab} fragment and the Nogo receptor

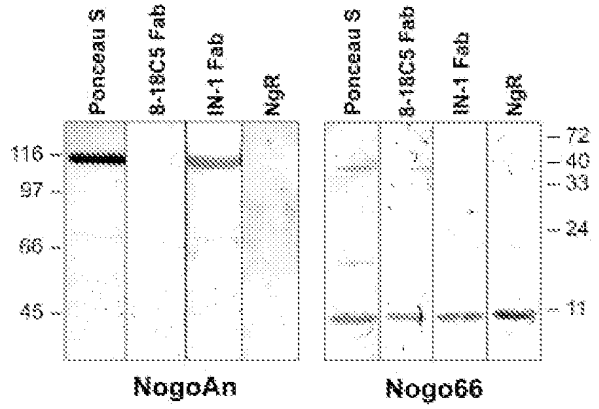

Figure 4. Binding activities of recombinant F_{ab} fragments as well as the recombinant NgR fusion protein for the bacterially produced NogoAn (left) and Nogo66 (right) on a Western blot. Recombinant NogoAn was purified from the periplasmic cell extract of *E. coli* via the *Strep*-tag II and applied to 8% w/v SDS-PAGE. Nogo66 was purified via the His_6 tag from solubilized inclusion bodies, refolded, and applied to 15% w/v SDS-PAGE. After electroblotting, the membranes were cut into two sets of stripes corresponding to lanes with identical samples. These were then either stained with Ponceau S or incubated with 100 nM solutions of the purified and digoxigenin-labeled recombinant F_{ab} fragments or the recombinant NgR fusion protein (TrxA-NgRΔ), followed by detection with an anti-digoxigenin F_{ab} fragment AP conjugate using BCIP and NBT as chromogenic substrates.

fusion protein merely showed weak binding activity towards NogoAn. Especially in the case of TrxA-NgRΔ the signals were barely above the background as determined with BSA. However, in the ELISA with Nogo66 as antigen strong binding activity was observed for the IN-1 F_{ab} fragment and for TrxA-NgRΔ. Notably, the lysozyme-specific D1.3 F_{ab} fragment (Skerra, 1994a), which was used as an additional control (data not shown), just showed background binding activity in this case, thus confirming the specificity of this assay as a whole.

In order to determine the dissociation constant for the interaction between the Nogo66 epitope peptide IKELRRL and TrxA-NgRΔ as well as the IN-1 F_{ab} fragment in solution, fluorescence titration experiments were performed. Defined amounts of the synthetic peptide were added to a solution of the purified proteins and the combined Tyr and Trp fluorescence emission was followed (Figure 6). A significant quenching effect was observed in the case of the NgR fusion protein, whereby the curve fit of the data according to the Law of Mass Action revealed a dissociation constant of $10.3 \pm 0.7\,\mu M$, a value not untypical for linear epitope peptides. In case of the IN-1 F_{ab} fragment the quenching effect was of lesser extent, indicating weaker binding activity for the same epitope peptide.

DISCUSSION

Despite several studies that have addressed the interactions between the CNS myelin protein Nogo-A and its presumed physiological receptors using the methods of neuro- or cell biology (Woolf and Bloechlinger, 2002; McGee and

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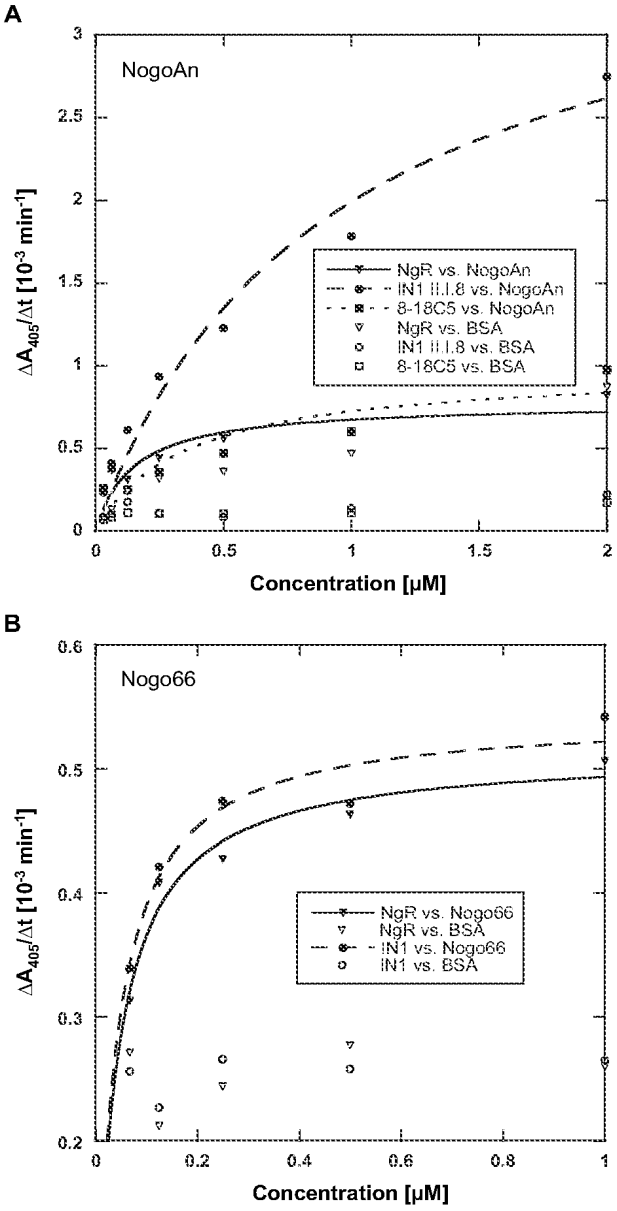

Figure 5. Binding activities of recombinant F_{ab} fragments as well as the recombinant NgR fusion protein for the bacterially produced NogoAn or Nogo66 in an ELISA. (A) A microtitre plate was coated with purified recombinant NogoAn (or just blocked with BSA), and purified digoxigenin-labeled F_{ab} fragments or TrxA-NgRΔ were applied in serial dilution. Bound protein was detected with an anti-digoxigenin F_{ab} fragment AP conjugate and *p*-nitrophenyl phosphate. (B) A microtitre plate was coated with purified recombinant Nogo66 (or just blocked with BSA) and probed in a similar manner as in (A).

Strittmatter, 2003; Schwab, 2004), a detailed characterization of these phenomena on the protein-chemical level has been missing. To this end, we have here attempted to apply the SPOT peptide array synthesis technique, which proved to be useful in the past for the characterization of protein interactions in many instances (Frank, 2002).

Cell biological studies have shown (Fournier *et al.*, 2001; Fournier *et al.*, 2002) that the inhibitory activity of the C-terminal region of Nogo-A, in particular Nogo66, is mediated by NgR. Our observations that the recombinant NgR fusion protein, TrxA-NgRΔ, binds to the bacterially expressed Nogo66 fragment on the Western blot and also in ELISA supports these findings. Using the SPOT array of peptides covering the complete Nogo-A primary structure as well as subsequent substitutional and length analysis, we were able to identify a linear binding site with the seven amino acid core peptide sequence 'IKxLRRL' ($x \neq P$).

This sequence is a central part of the Nogo66 domain (IKELRRL, residues 49–55, corresponding to sequence positions 1103–1109 within the full length human Nogo-A protein). IKELRRL is also part of the peptide Pep4, corresponding to residues 31–55 of Nogo66, which was previously shown to exert growth cone-collapsing and outgrowth-inhibiting activity at a concentration of 4 μM (GrandPre *et al.*, 2000). However, the inhibitory potency of this peptide was much lower than that of the entire Nogo66 fragment.

Therefore, it was suggested that other regions within this domain contribute to its apparent affinity in the nanomolar range. Indeed, the peptide NEP1-40 (residues 1–40 of Nogo66) was identified, which binds NgR expressed on COS7 cells and blocks Nogo66 or CNS myelin inhibition of axonal outgrowth *in vivo* (GrandPre *et al.*, 2002). Notably, in our SPOT experiment we could not identify a second binding site for NgR around the N-terminus of the Nogo-66 region. Thus, the lower inhibitory activity observed for Pep4 could be due to a loss of conformational stabilization by more adjacent residues present in NEP1-40 or Nogo66, which are also absent in the 15 mer peptides investigated here.

Fluorescence titration with TrxA-NgRΔ and the synthetic Nogo66 epitope peptide revealed a K_D value around 10 μM for the interaction. This is considerably higher than the nanomolar values determined for the NgR-Nogo66 interaction in cell-based assays (Fournier *et al.*, 2001) but comparable to the value of 2 μM reported from measurements of the interaction between a Nogo66 alkaline phosphatase (AP) fusion and NgR displayed on ribosomes (Schimmele *et al.*, 2005). In other biochemical assays, such as co-immunoprecipitation experiments with the purified ligand-binding domain of NgR used for crystallization and a Nogo66-AP fusion (He *et al.*, 2003), the mutual affinity also appeared to be weaker than the low nM value estimated from the cell-based experiments.

In our SPOT assay there was no indication of another binding site for the recombinant NgR fusion protein, TrxA-NgRΔ, within the N-terminal region of Nogo-A, NogoAn. This finding is in agreement with the lack of binding activity towards the bacterially produced NogoAn fragment on the Western blot and in an ELISA and is also supported by a previous study (Oertle *et al.*, 2003b). An interaction between the N-terminal region of Nogo-A and NgR was only reported once (Hu *et al.*, 2005). These authors applied fusion proteins between different N-terminal fragments of Nogo-A and alkaline phosphatase to the full length NgR expressed on COS-7 cells and identified an N-terminal subregion, dubbed Nogo-A-24 (residues 995–1018), which bound to the NgR ectodomain with nanomolar affinity.

In the present study, we deliberately omitted Nogo-A-24 from our bacterially produced NogoAn fragment because this sequence stretch is located at the very C-terminus of the

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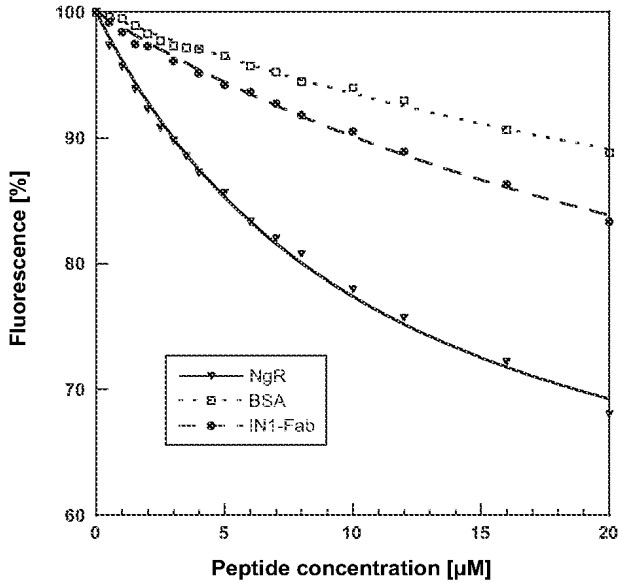

Figure 6. Fluorescence titration of the recombinant NgR fusion protein, TrxA-NgRΔ, and of the IN-1 F_{ab} fragment with the synthetic Nogo66 epitope peptide oAbz-TIKELRRLFL-NH_2. Fluorescence of the proteins, all at a concentration of 1 μM in PBS, was excited at 280 nm and detected at 340 nm. BSA served as negative control. Binding of the peptide carrying an N-terminal *o*-aminobenzoyl group (Abz) as chromophore leads to a quenching effect on the Tyr and Trp fluorescence of the target protein upon complex formation (Voss and Skerra, 1997). In the case of TrxA-NgRΔ curve fit of the data resulted in an asymptotic value of 52% (i.e., corresponding to $Q_{max} = 48\%$ maximal quenching).

region specific for the Nogo-A splice variant (Hu *et al.*, 2005), which thus might explain the apparent absence of binding activity for the NgR fusion protein on Western blot and in ELISA. However, no binding activity between TrxA-NgRΔ and peptides corresponding to the Nogo-A-24 region could be detected in the SPOT epitope mapping experiments either (cf. peptide no. 332, residues 995–1009; Figure 2D), thus calling a strong interaction as postulated into question. In addition, cell biological experiments clearly demonstrated that the inhibitory action of N-terminal Nogo-A is NgR-independent (Oertle *et al.*, 2003b). Hence, a second binding site for NgR in Nogo-A remains elusive.

We could also not define a linear epitope for either the IN-1 F_{ab} fragment or its engineered version II.I.8 within the N-terminal extramembrane region of Nogo-A, NogoAn. Normally, the SPOT technique works reliably for the identification of non-conformational epitopes due to the high local density of peptide molecules that are chemically immobilized on the membrane. In particular, this method has successfully served to identify contact sites for a variety of protein complexes (Reineke *et al.*, 2001; Reineke, 2004).

On the other hand we were able to demonstrate specific binding of the IN-1 F_{ab} fragment to NogoAn, expressed as a soluble protein fragment in *E.coli*, on the Western blot. Its engineered version II.I.8 also served nicely to detect NogoAn in an ELISA. This is consistent with earlier findings for the bacterially expressed, highly homologous N-terminal region of Nogo-A from rat (Fiedler *et al.*, 2002). Therefore, the failure to identify a defined epitope peptide for the IN-1 F_{ab} fragment may reflect a strong conformational dependence of an overall rather weak antibody-antigen interaction. This interpretation is supported by a nearly complete loss of inhibitory activity of Nogo-A or its fragments in the presence of 8 M urea as described by others (Oertle *et al.*, 2003b). Nevertheless, interactions between the IN-1 F_{ab} fragment and peptides derived from NogoAn leading to weak signals on the peptide array (cf. Figure 1A, e.g., spots no. 59 and 222) might indicate discontinuous stretches of an epitope that depends on the correct folding of the entire protein. However, this interpretation remains hypothetical unless proven by three-dimensional structure.

Notably, while our data support the role of the IKELRRL sequence within the Nogo66 domain as a potential binding site for the Nogo receptor, its interaction was not specific to the NgR fusion protein. In our SPOT assays the partially humanized mouse F_{ab} fragment IN-1, its engineered version II.I.8, and the MOG specific F_{ab} fragment 8-18C5 all bound to the Nogo66 peptides no. 366–367, covering the same IKELRRL core epitope. Furthermore, interaction with the bacterially produced Nogo66 fragment was subsequently demonstrated for the IN-1 F_{ab} fragment, and also for 8-18C5 (not shown), via Western blot and ELISA.

These findings raise the question whether the IN-1 antibody with its well established Nogo-A neutralising activity (Schwab, 2004) could also act through blockade of an interaction between Nogo-A and NgR. However, the observed cross-reactivity of the unrelated antibody 8-18C5 with the IKELRRL epitope sequence argues against this hypothesis. In fact, it is surprising that the same peptide shows such a strong and, within the entire Nogo-A sequence, essentially unique binding activity both for NgR, the IN-1 and the 8-18C5 F_{ab} fragments. Hence, one might speculate that this behaviour corresponds to some non-specific binding activity of this peptide when it is removed from its folded protein context.

In a recent NMR structural analysis of the first 60 residues of Nogo66, the IKELRRL sequence was identified as one of the four exposed positively charged patches that could be candidates for binding to NgR (Li *et al.*, 2006). Interestingly, the identified epitope also occurs in other human proteins, for example in the fibroblast growth factor 4 (FGF4). In its crystal structure, the sequence IKRLRRL forms an exposed stretch at the N-terminus of the β-trefoil core domain and is presumably involved in FGF receptor binding (Bellosta *et al.*, 2001). Even though the biological implications of the multiple interactions of the IKxLRRL motif are not clear at present, its mechanisms of interaction with NgR—or with the IN-1 F_{ab} fragment—in conjunction with neuronal regeneration certainly deserve further investigation.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft (research grant no. SK 33/2-3,4).

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DOI: 10.1002/jmr

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DOI: 10.1002/jmr